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Nuclear Gene 33/Mig6 regulates the DNA damage response through an ATM serine/threonine kinase–dependent mechanism

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Gene 33 (Mig6, ERFF1) is an adaptor protein with multiple cellular functions. We recently linked Gene 33 to the DNA damage response (DDR) induced by hexavalent chromium (Cr(VI)), but the molecular mechanism remains unknown. Here we show that ectopic expression of Gene 33 triggers DDR in an ATM serine/threonine kinase (ATM)–dependent fashion and through pathways dependent or not dependent on ABL proto-oncogene 1 non-receptor tyrosine kinase (c-Abl). We observed the clear presence of Gene 33 in the nucleus and chromatin fractions of the cell. We also found that the nuclear localization of Gene 33 is regulated by its 14-3-3–binding domain and that the chromatin localization of Gene 33 is partially dependent on its ErbB-binding domain. Our data further indicated that Gene 33 may regulate the targeting of c-Abl to chromatin. Moreover, we observed a clear association of Gene 33 with histone H2AX and that ectopic expression of Gene 33 promotes the interaction between ATM and histone H2AX without triggering DNA damage. In summary, our results reveal nuclear functions of Gene 33 that regulate DDR. The nuclear localization of Gene 33 also provides a spatial explanation of the previously reported regulation of apoptosis by Gene 33 via the c-Abl/p73 pathway. On the basis of these findings and our previous studies, we propose that Gene 33 is a proximal regulator of DDR that promotes DNA repair.

Gene 33 is an inducible adaptor protein containing multiple domains for protein–protein interaction and signal transduction (Fig. 1A). These include a CRIB domain for interaction with small GTPases of the Rho family, a 14-3-3–binding domain (14-3-3BD), several proline-rich SH3³-binding domains,

an SH2-binding domain, and a PDZ-binding domain (1–5). A region highly homologous to the non-receptor-tyrosine kinase-activated CDC42-associated kinase 1 (ACK-1) is located at the C-terminal half of the protein (6), within which is an ErbB-binding domain (EBD) (7–10). There are two stretches of PEST sequences, suggesting that Gene 33 is a potential target for proteasome-mediated protein degradation (11). A putative DEAD box is located in the N-terminal half of the protein (12). A putative nuclear localization sequence (NLS) is located close to the C terminus of the protein (13).

The most functionally significant domain of Gene 33 characterized thus far is its EBD. EBD of Gene 33 has been well-characterized to be required for binding of Gene 33 to EGFR and c-Abl (8–10, 14). Gene 33 interacts with the kinase domain of EGFR, thereby inhibiting its kinase activity (10). Binding of Gene 33 to the kinase domain of c-Abl, on the other hand, elevates its kinase activity (14). Gene 33 interacts with kinase domains of EGFR and c-Abl through its EBD in an apparently very similar fashion, as these domains are structurally close (14). Several residues within the EBD, including Tyr-358, are critical for its binding to EGFR and c-Abl (10, 14). Mutation of Tyr-358 to alanine (Y358A) significantly disrupts the interaction of Gene 33 with EGFR or c-Abl (Fig. 1A) (10, 14).

Gene 33 is an immediate early response protein that can be induced by a wide array of mitogenic and stress stimuli, including ligands for receptors of the EGFR family (9, 15–18). Thus, Gene 33 is viewed as a feedback inhibitor of the EGFR signaling pathway (15, 19). Accumulating evidence also indicates that Gene 33 promotes apoptosis and senescence (14, 17, 20, 21). The pro-apoptotic function of Gene 33 was first observed in rat neonatal cardiomyocytes, where overexpression of Gene 33 induces dramatic apoptosis (17). Depletion of Gene 33 by RNAi reduces apoptosis induced by acute hypoxia in part through inhibition of the EGFR-mediated activation of the pro-survival pathway in these cells (17). A later work showed that Gene 33 facilitates mammary epithelial cell apoptosis resulting from EGF deprivation (14). This is accomplished by activating the protein-tyrosine kinase c-Abl through direct interaction with

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³ The abbreviations used are: SH3, Src homology 3; SH2, Src homology 2; 14-3-3BD, 14-3-3–binding domain; EBD, ErbB-binding domain; NLS,

nuclear localization sequence; EGFR, EGF receptor; DDR, DNA damage response; IP, immunoprecipitation; P-, phosphorylated; Ni-NTA, nickel-nitrilotriacetic acid; MOI, multiplicity of infection.

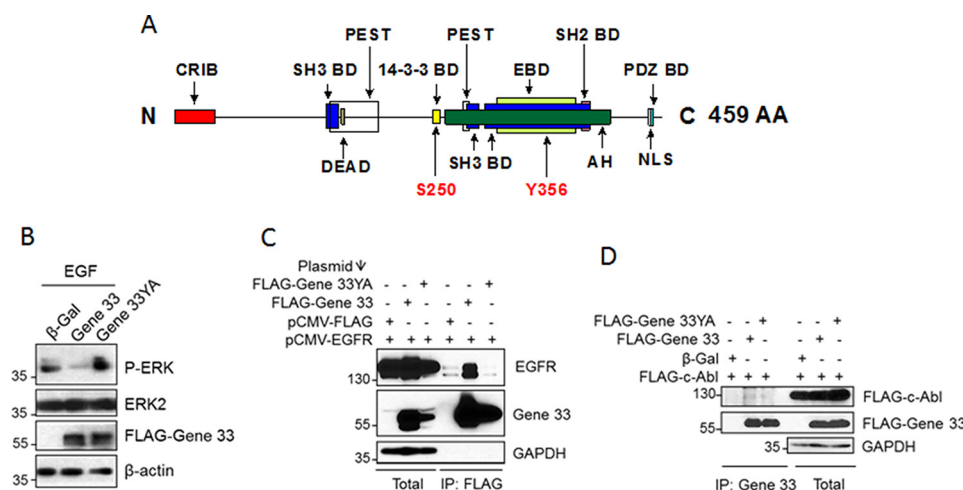


Figure 1. Ectopic expression of wild-type but not the Y356A mutant Gene 33 inhibits EGFR signaling and induces apoptosis. A, linear representation of rat Gene 33 domain structure highlighting Tyr-356 (Y356) within EBD and Ser-250 (S250) with 14-3-3BD. B, A549 cells growing at 80% confluence were infected with adenoviral vectors at 50 MOI under serum starvation for 24 h. Cells were then left untreated or treated with EGF (50 ng/ml) for 10 min before harvesting for Western blotting using antibodies for phospho-ERK, ERK-2, FLAG, and β -actin (as loading control), as indicated. C, HEK293T cells were transfected with the indicated plasmids and harvested 24 h later for immunoprecipitation with an anti-FLAG antibody. The total lysates and immunoprecipitants were then subjected to Western blotting with the indicated antibodies on the right. D, BEAS-2B cells were transfected with the FLAG-tagged c-Abl plasmid followed by infection with the indicated adenoviral vectors 24 h after transfection. The cells were then harvested 24 h later for immunoprecipitation with the anti-Gen 33 antibody. The total lysates and immunoprecipitants were then subjected to Western blotting with an anti-FLAG antibody (to detect both FLAG-Gen 33 and FLAG-c-Abl) and GAPDH.

its kinase domain, which subsequently phosphorylates and activates the p73 tumor suppressor (14). A pro-apoptotic role of Gene 33 has also been reported in pancreatic islet β -cells, where it promotes apoptosis induced by the endoplasmic reticulum stress and DNA-damaging agents (21, 22). Interestingly, Gene 33 has also been shown to be anti-apoptotic in breast cancer cells and to have no effect on apoptosis in cortical neurons (23, 24). Moreover, transgenic expression of Gene 33 in mouse cardiomyocytes does not trigger significant apoptosis (25). Thus, the effect of Gene 33 on cellular apoptosis appears to be cell type- and dose-dependent.

Our recent work revealed a novel connection between Gene 33 and the DNA damage response (DDR) (26). We found that Gene 33 depletion increases DNA damage, chromosome instability, and cell transformation induced by genotoxic Cr(VI) in lung epithelial cells (26). In the present study, we investigated the mechanism underlying this novel observation. We report herein surprising nuclear and chromatin localization of Gene 33, which is in sharp contrast with the current view that Gene 33 is an exclusively cytoplasmic protein. We provide evidence that Gene 33 may regulate DDR at the chromatin level. Our results also provide a spatial explanation on the previously reported regulation of apoptosis by Gene 33 via the c-Abl/p73 pathway.

Results

Ectopic expression of wild-type but not the Y356A mutant Gene 33 inhibits EGFR signaling

We constructed an adenoviral vector expressing FLAG-tagged rat Gene 33 containing the Y356A mutation (equivalent to Y358A of human Gene 33), a critical residue required for optimal binding of Gene 33 to EGFR or c-Abl (10, 14). Fig. 1A illustrates the linear structure of rat Gene 33 with the point mutation sites relevant to this study indicated. To characterize

the adenoviral vector encoding this Gene 33 mutant, we compared the effect of this vector on A549 lung carcinoma cells with those of the adenoviral vectors expressing β -Gal (as a negative control) and wild-type Gene 33 (17). As shown in Fig. 1B, ectopic expression of wild-type Gene 33 (Gene 33) but not the mutant Gene 33 (Gene 33YA) largely prevented the phosphorylation of ERK induced by EGF, consistent with the inhibitory role of Gene 33 on the EGFR signaling pathway (8, 9, 14, 17). Fig. 1 (C and D) shows that Gene 33YA had reduced interactions with EGFR and c-Abl compared with wild-type Gene 33 in co-immunoprecipitation (co-IP) experiments, in agreement with the previous findings that the mutated residue is critical for interaction with EGFR and c-Abl (10, 14).

Gene 33 promotes DDR and apoptosis through largely distinct mechanisms

Our recent study revealed a potential involvement of Gene 33 in DDR. We found that Gene 33 depletion elevates Cr(VI)-induced DDR (26). To further determine the effect of Gene 33 on DDR, we examined the effect of ectopic expression of Gene 33 and Gene 33YA on DDR. We observed that adenoviral vector-mediated overexpression of either Gene 33 or Gene 33YA in both A549 and BEAS-2B cells significantly elevated the levels of γ H2AX and p53 phosphorylation at serine 15 (P-p53), indicating activation of DDR (Fig. 2, A and B). Strikingly, only wild-type Gene 33, but not Gene 33YA, strongly induced apoptosis, as indicated by induction of cleaved caspase-3 (Fig. 2, A and B). These data indicate that ectopically expressed Gene 33 induces apoptosis in an EBD-dependent manner and DDR in an EBD-independent fashion. Notably, p53 was equally activated by Gene 33 and Gene 33YA, suggesting that Gene 33-induced apoptosis is p53-independent. Note that A549 cells showed a very low basal P-p53 level, which was significantly elevated by ectopic expression of Gene 33, whereas BEAS-2B cells had a

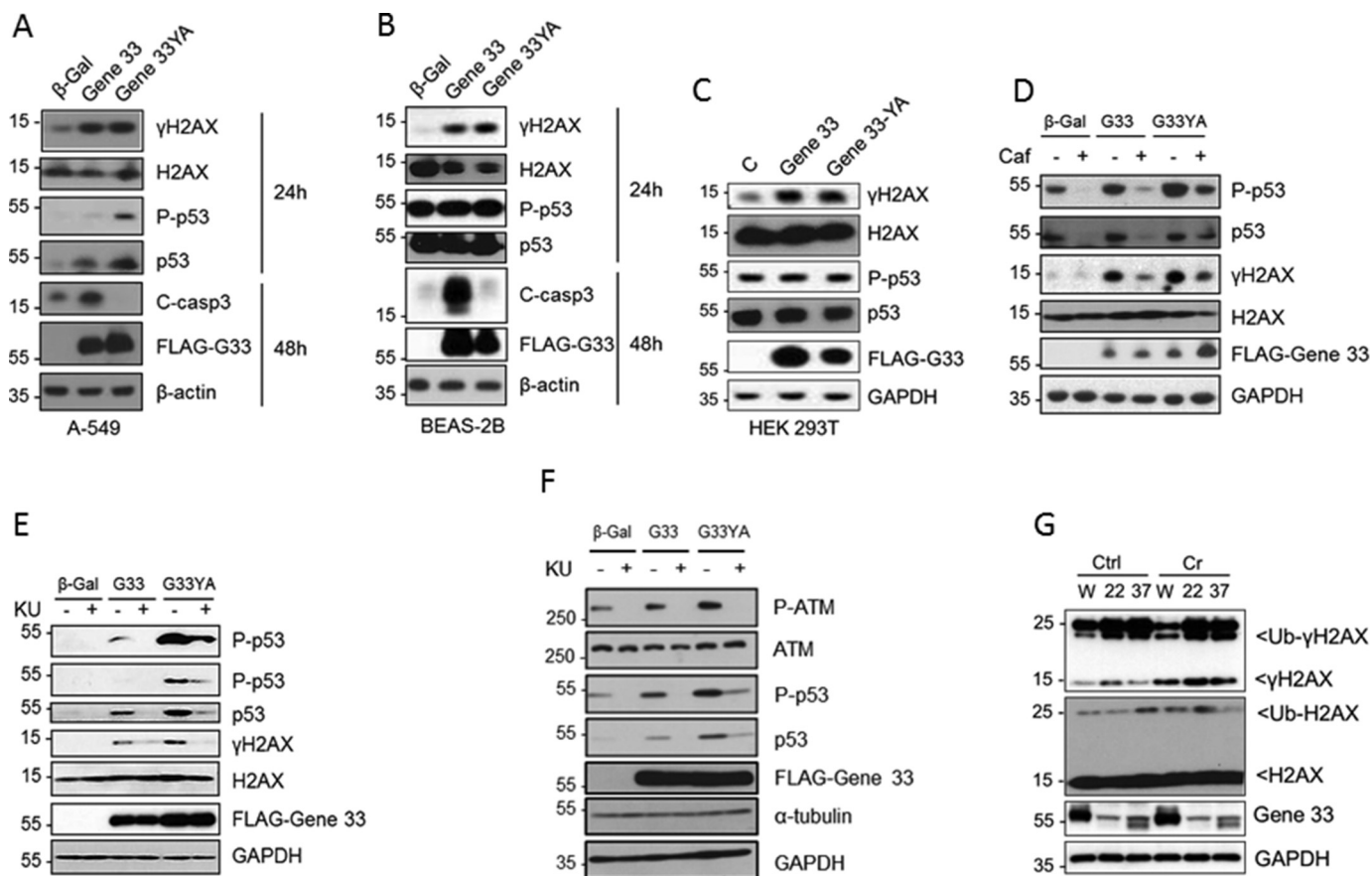


Figure 2. Gene 33 promotes DDR and apoptosis through distinct mechanisms. A, A549 cells were infected with the indicated adenoviral vectors at 50 MOI. Cells were harvested 24 or 48 h (as indicated) postinfection for Western blotting using the indicated antibodies (P-p53, phospho-p53-serine 15; p53, total p53; γ H2AX, phospho-histone H2AX at serine 139; H2AX, total H2AX; C-casp3, cleaved caspase-3). B, the experiment was performed as in A using BEAS-2B cells. C, HEK293T cells were transfected with plasmids for empty p-CMV5-FLAG, p-CMV5-FLAG-Gene 33, or p-CMV5-FLAG-Gene 33YA followed by harvesting 48 h later for Western blotting to detect the proteins indicated on the right. D, A549 cells were infected with the indicated adenoviral vectors at 50 MOI and harvested 24 h later with or without treatments with caffeine (10 mM) for the last 4 h before harvesting. Western blotting was then performed to detect the proteins indicated on the right. E and F, the experiment was performed as in D with KU55933 (10 μ M) replacing caffeine. G, two clones of BEAS-2B cell lines (C1 and C2) with gene 33 depletion using the CRISPR/Cas9 system and a control BEAS-2B cell line (W) were either left untreated or treated with 5 μ M Cr(VI) for 6 h. Cells were then harvested for Western blotting to detect the indicated proteins.

much higher basal level of P-p53, which exhibited limited increase upon ectopic expression of Gene 33. This discrepancy was probably a result of the compensatory effect from the presence of SV40 large T antigen, an inhibitor of p53, in BEAS-2B cells (27). This observation further supports the notion that Gene 33 triggers apoptosis with limited involvement of p53 in these cells.

To exclude the possibility that the effect of Gene 33 on DDR was a result of the adenoviral vector *per se*, we ectopically expressed Gene 33 or Gene 33YA using plasmid DNA in HEK293T cells by conventional transfection. We observed similar effects of Gene 33 and Gene 33YA on γ H2AX as in A549 and BEAS-2B cells (Fig. 2C). Similar to BEAS-2B cells, the basal level of P-p53 was high in HEK293T cells and was not appreciably elevated by ectopic expression of either wild-type Gene 33 or Gene 33YA (Fig. 2C). As in BEAS-2B cells, HEK293T cells also contain SV40 large T antigen.

DDR is mediated by ATM family protein kinases that are activated upon DNA damage (28). Activation of these kinases leads to phosphorylation of histone H2AX at the serine 139 position to produce γ H2AX, a hallmark of DDR, and P-p53 (28, 29). To determine whether DDR triggered by ectopic Gene 33

expression is mediated by ATM/ATR, we treated A549 cells expressing wild-type Gene 33 or Gene 33YA with caffeine, a known inhibitor of ATM/ATR (30), before harvesting the cells for Western blotting. Our data showed that caffeine largely prevented the increase in P-p53 and γ H2AX induced by ectopic expression of either wild-type Gene 33 or Gene 33YA (Fig. 2D), indicating that DDR elicited by Gene 33 is indeed ATM/ATR-dependent. We next used KU55933, a specific ATM kinase inhibitor (31), to repeat the experiment in Fig. 2D. As shown in Fig. 2 (E and F), KU55933 strongly inhibited DDR induced by either wild-type Gene 33 or Gene 33YA. Thus, ATM is probably the kinase that mediates Gene 33-induced DDR. Note that the ATM kinase activity, manifested as phosphorylation of its serine 1981 (28), was elevated by wild-type Gene 33 or Gene 33YA and dramatically reduced by KU55933 (Fig. 2F).

We have shown previously that Gene 33 depletion by RNAi leads to elevated DDR in response to Cr(VI) (26). To confirm this, we used a CRISPR/Cas9-based system to deplete Gene 33 in BEAS-2B cells and examined the effect on DDR. As shown in Fig. 2G, reduced Gene 33 levels led to increased DDR in response to Cr(VI) in two CRISPR/Cas9 cell clones compared with the wild-type clone (Fig. 2G). Note that Gene 33 depletion

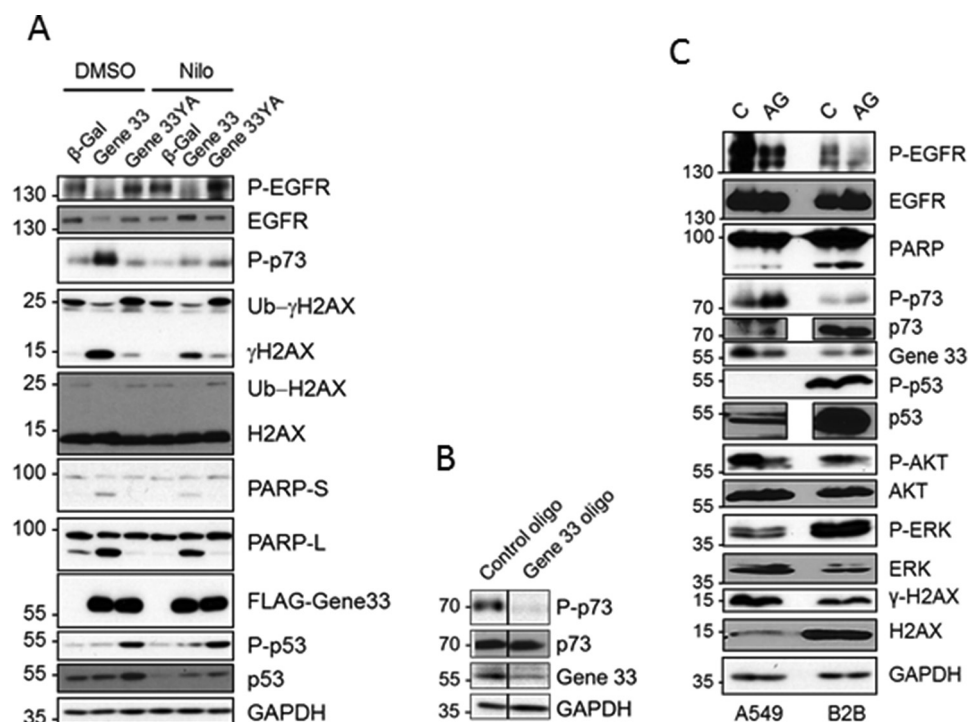


Figure 3. Gene 33 promotes apoptosis via both c-Abl/p73 and EGFR/AKT pathways. A, A549 cells were infected with adenoviral vectors at 50 MOI and treated with DMSO (as vehicle) or Nilotinib (2 μ M) for 48 h before harvesting for Western blotting to detect the proteins indicated on the right (P-EGFR, phospho-EGFR; P-p73, phospho-p73; PARP, PARP1). B, A549 cells were transfected with either a scrambled siRNA oligonucleotide (Control oligo) or an siRNA oligonucleotide for Gene 33. Cells were harvested 48 h later for Western blotting to detect the proteins indicated on the right. C, A549 and BEAS-2B cells were treated with either DMSO (vehicle) or AG1478 (10 μ M) for 48 h before harvesting for Western blotting to detect the proteins indicated on the right.

elevated the levels of both the basal and Cr(VI)-induced γ H2AX, particularly ubiquitinated γ H2AX (Fig. 2G).

Gene 33 promotes apoptosis via both c-Abl/p73 and EGFR/AKT pathways

A previous study has shown that Gene 33 is able to interact and activate non-receptor tyrosine kinase c-Abl, thereby inducing mammary epithelial cell apoptosis resulting from EGFR inactivation (14). c-Abl promotes apoptosis by phosphorylating and stabilizing p73, a p53 family tumor suppressor (32, 33). To test whether a similar mechanism is involved in the induction of apoptosis by Gene 33 in lung carcinoma cells, we ectopically expressed either wild-type Gene 33 or Gene 33YA using adenoviral vectors with or without the presence of the specific c-Abl inhibitor nilotinib (34). We found that wild-type Gene 33 but not Gene 33YA induced strong phosphorylation of p73 at tyrosine 99 (P-p73), a target of c-Abl (35) (Fig. 3A). Phosphorylation of p73 was accompanied by a significantly increased level of cleaved PARP1, a common marker of apoptosis (Fig. 3A). Inhibition of c-Abl by nilotinib largely prevented p73 phosphorylation and reduced the level of cleaved PARP1 induced by wild-type Gene 33 (Fig. 3A). In contrast, Gene 33YA elicited levels of increase in γ H2AX (when combining ubiquitinated and non-ubiquitinated forms) and P-p53 equivalent to those elicited by Gene 33 without notably elevating the level of P-p73 and cleaved PARP1. Importantly, although nilotinib could partially prevent the increase in the γ H2AX level induced by wild-type Gene 33, it had limited effect on that induced by Gene 33YA (Fig. 3A). These data indicate that induction of the DDR by Gene 33 is mediated by both c-Abl-dependent and c-Abl-inde-

pendent pathways. As expected, wild-type Gene 33 but not Gene 33YA inhibited EGFR tyrosine phosphorylation, in line with the previous observations (9, 17). Furthermore, knock-down of Gene 33 alone with siRNA dramatically reduced the level of P-p73 (Fig. 3B). These data, combined with those in Fig. 2, demonstrate that Gene 33 promotes apoptosis through a c-Abl/p73-dependent pathway, whereas it activates DDR via both c-Abl-dependent and -independent pathways.

Previous work has shown that Gene 33, as an inhibitor of EGFR, can promote apoptosis by inhibiting the survival pathway downstream of EGFR in cardiomyocytes (17). To confirm this in lung epithelial and lung cancer cells, we inhibited EGFR using AG1478, a specific EGFR kinase inhibitor, in BEAS-2B and A549 cells. We found that inhibition of the EGFR, manifested as a reduced level of EGFR tyrosine phosphorylation, significantly elevated the levels of P-p73 and cleaved PARP1 without affecting the levels of γ H2AX and P-p53 in both cell types (Fig. 3C). The elevated level of P-p73 upon EGFR inhibition was probably caused by increased binding of Gene 33 with c-Abl resulting from the reduced EGFR activity, as described previously (14). Furthermore, EGFR inhibition reduced the level of P-AKT but not P-ERK (Fig. 3C). These data suggest that both activation of the p73 pathway and inhibition of the AKT pathway may contribute to apoptosis induced by EGFR inhibition. Because Gene 33 is a known EGFR inhibitor, our data support the notion that Gene 33 promotes apoptosis through both activation of the c-Abl/p73-mediated pro-apoptotic pathway and inhibition of the EGFR/AKT-mediated pro-survival pathway. Our data also

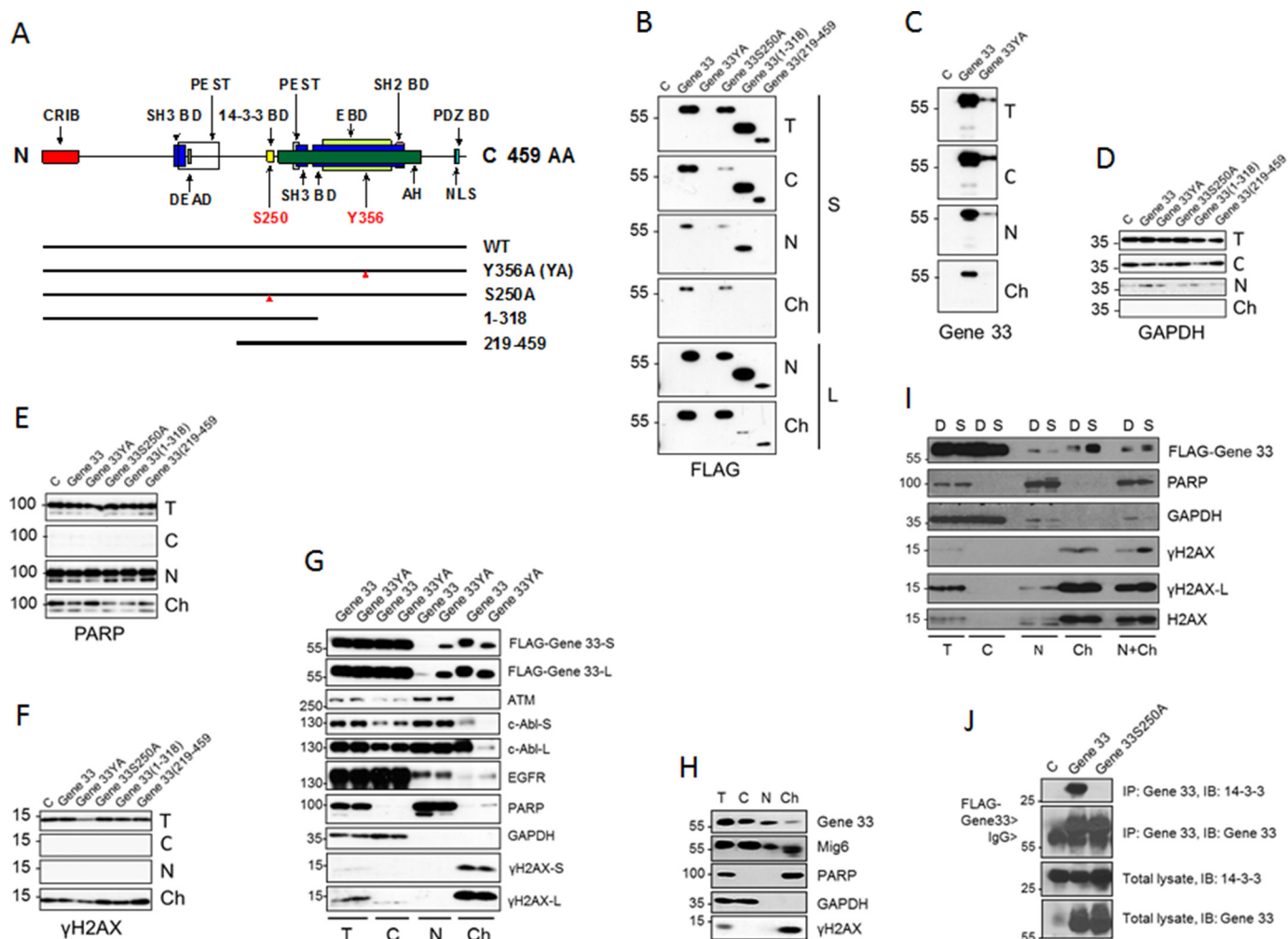


Figure 4. Gene 33 is partially localized in the nucleus, is associated with chromatin, and regulates chromatin localization of c-Abl. A, schematic representation of the Gene 33 mutants used in B–F. B–F, BEAS-2B cells were transfected with the indicated FLAG-tagged Gene 33 mutants. Cell fractionation was carried out as described under “Experimental procedures.” Proteins in different cellular fractions were subjected to Western blotting with antibodies indicated at the bottom of each panel (anti-FLAG antibody for FLAG-tagged Gene 33). L, long exposure; S, short exposure; T, total lysate; C, cytoplasmic; N, nuclear (excluding chromatin); Ch, chromatin. G, A549 cells were infected with adenoviral vectors expressing FLAG-tagged wild-type Gene 33 or Gene 33YA. Cell fractionation was carried out as described under “Experimental procedures” 24 h later. Proteins in the cellular fractions were subjected to Western blotting to detect proteins indicated on the right. H, cell fractionation was carried out in BEAS-2B cells. Proteins in the cellular fractions were subjected to Western blotting to detect the proteins indicated on the right. I, A549 cells were infected with adenoviral vectors expressing FLAG-tagged wild-type Gene 33 for 24 h. Cells were treated with either DMSO (D) or 2.5 μ M Chk1 inhibitor SB218078 (S) for 4 h before harvest. Cell fractionation was then carried out as described under “Experimental procedures.” Proteins in the cellular fractions were subjected to Western blotting to detect proteins indicated on the right. J, HEK293T cells were transfected with the indicated FLAG-tagged Gene 33 constructs followed by immunoprecipitation with an anti-Gen 33 antibody 24 h later. The products of immunoprecipitation and total cell lysates were then subjected to Western blotting with anti-Gen 33 or anti-14-3-3 antibodies as indicated.

indicate that inhibition of EGFR alone is not sufficient to induce DDR in these cells.

Gene 33 is partially localized in the nucleus, is associated with chromatin, and regulates chromatin localization of c-Abl

The association of Gene 33 with DDR, as indicated by the present work and a previous study (26), raises the probability that Gene 33 may have nuclear localization, as most of the mediators of DDR are in the nucleus (36). The ability of Gene 33 to interact with c-Abl that phosphorylates p73 also implies probable nuclear presence of this protein, because, whereas c-Abl can be both cytoplasmic and nuclear (37), p73 is mainly a nuclear protein (38). Moreover, Gene 33 does contain a putative NLS (Fig. 1A). To determine this, we transfected plasmids encoding FLAG-tagged wild-type Gene 33 and various Gene 33

mutants (Fig. 4A) into BEAS-2B cells followed by a cell fractionation assay to examine the subcellular presence of these Gene 33 mutants. Our results showed that whereas wild-type Gene 33 was mainly located in the cytoplasmic fraction, it could also be detected in the nuclear and the chromatin fractions (Fig. 4B, lane 2). The Gene 33 mutant containing a point mutation on the key serine residue within its 14-3-3 domain (Gene 33(S250A); Figs. 1A and 4A), which prevents its phosphorylation and its interaction with the adaptor protein 14-3-3 (39), exhibited a significantly reduced level in the cytoplasmic fraction (Fig. 4B, lane 4). The deletion mutant missing the C-terminal part of Gene 33 (containing the EBD) but retaining the 14-3-3BD (Gene 33(1–318); Fig. 4A) showed a dramatically reduced level in the chromatin fraction (Fig. 4B, lane 5). The deletion mutant of Gene 33 missing the N-terminal portion of the pro-

tein but retaining the EBD and the 14-3-3BD (Gene 33(219–459)) had a subcellular distribution similar to that of wild-type Gene 33 (Fig. 4B, *last lane*).

Because we were unable to detect the expression of Gene 33YA mutant (Gene 33YA) using the anti-FLAG antibody (as a result of the low expression level of this mutant in BEAS-2B cells) (Fig. 4B, *lane 3*), we used the anti-Gene 33 antibody (with a higher sensitivity toward FLAG-tagged Gene 33 than that of the anti-FLAG antibody) to detect this mutant. As shown in Fig. 4C, we were able to detect Gene 33YA, albeit at a much lower level as compared with wild-type Gene 33. Of note, the detected Gene 33YA is not endogenous Gene 33, as it did not appear in the control group (Fig. 4C, *lane 1*). Notably, Gene 33YA appears to have proportionally less presence in the chromatin fraction (Fig. 4C). This result suggests that EBD may be important for chromatin association of Gene 33, in agreement with the distribution of Gene 33(1–318). Fig. 4 (D–F) shows the quality of the cell fractionation as indicated by the presence or absence of GAPDH, a mainly cytosolic protein; PARP1, a mostly nuclear protein and chromatin-binding protein; and histone H2AX (γ H2AX), an exclusive chromatin-associated protein. It is also noteworthy that the putative NLS located at the very C terminus of Gene 33 (Figs. 1A and 4A) does not seem to be important for the nuclear localization of Gene 33, as Gene 33(1–318), which is devoid of the NLS (Fig. 4A), had a rather robust nuclear presence (Fig. 4B).

We next examined whether nuclear and chromatin localization of ectopically expressed Gene 33 also occurs in A549 cells. We ectopically expressed wild-type Gene 33 and Gene 33YA using adenoviral vectors in A549 cells and then performed the cell fractionation assay. As shown in Fig. 4G, these two proteins all appeared in the nuclear and chromatin fractions. Notably, most of wild-type Gene 33 was in the chromatin fraction as compared with much more of Gene 33YA in the soluble nuclear fraction (Fig. 4G, *lanes 5–8*), despite the fact that the levels of these two proteins were similar in both total lysates and cytoplasmic fractions (Fig. 4G, *lanes 1–4*). This observation agrees with the data from BEAS-2B cells, where Gene 33YA and Gene 33(1–318) both had an impaired EBD domain and had much lower levels in the chromatin fraction than in the nuclear fraction compared with wild-type Gene 33. These data confirm that the EBD motif of Gene 33 is important for its chromatin targeting. Note that significant cleavage of PARP1 occurred in cells expressing wild-type Gene 33 but not Gene 33YA (Fig. 4G, *lane 5*), confirming that only wild-type Gene 33 induces significant apoptosis. Interestingly, a much higher level of c-Abl was observed in the chromatin fraction of the cells expressing wild-type Gene 33 than in those expressing Gene 33YA (Fig. 4G, *lanes 7 and 8*), suggesting that Gene 33 may regulate the targeting of c-Abl to the chromatin via its EBD. A cell fractionation experiment in BEAS-2B cells confirmed that the endogenous Gene 33 could also be detected in the nuclear and the chromatin fractions using both the anti-Gene 33 antibody and a commercial anti-Mig6 antibody (Fig. 4H).

Binding to 14-3-3 through phosphorylated 14-3-3BD is a common mechanism for many proteins to maintain cytoplasmic localization (40). It has been reported that the key serine residue (serine 250; Fig. 1A) within the 14-3-3BD of Gene 33

can be phosphorylated by the serine/threonine kinase Chk1 (39, 41). To determine the role of Chk1 in subcellular distribution of Gene 33, we checked the effect of SB218078, a specific Chk1 inhibitor. We found that inhibition of Chk1 in A549 cells led to a significant increase of ectopically expressed Gene 33 in the chromatin fraction (Fig. 4I). When the nuclear and chromatin fractions were combined, the total nuclear Gene 33 was still higher in the group with the Chk1 inhibitor (Fig. 4I, compare the *last two lanes*). These data indicate that phosphorylation of Gene 33 by Chk1 probably reduces its nuclear translocation. Note that the Chk1 inhibitor-treated group showed a higher level of γ H2AX, consistent with the previous observation that Chk1 inhibition increases the level of γ H2AX as a result of increase in replicative DNA damage (42). Our co-IP experiment confirmed that ectopically expressed Gene 33S250A mutant was not able to bind to ectopically expressed 14-3-3 δ (Fig. 4J).

To further demonstrate the nuclear presence of Gene 33, we transfected FLAG-tagged Gene 33 into BEAS-2B cells and examined the subcellular localization of the ectopically expressed Gene 33 using confocal microscopy after immunofluorescent staining with an anti-FLAG antibody. As shown in Fig. 5A, although the majority of FLAG signals were detected in the cytoplasm of cells, they were also detectable in the nucleus. Note that no FLAG signals were detected in the neighboring cells that did not take up the plasmid. We chose a cell with an apparently very low level of the nuclear FLAG signal for in-depth analysis with continuous optical sections. We found that even in this cell, nuclear FLAG signals were detectable at various levels in these sections (Fig. 5B). In another cell with a high level of the nuclear FLAG signal, strong FLAG signals were visible in all sections (Fig. 5C).

We next checked the subcellular localization of endogenous Gene 33 using immunofluorescence confocal imaging with the anti-Gene 33 antibody. Similar to ectopically expressed Gene 33, the majority of endogenous Gene 33 signals were detected in the cytoplasm of BEAS-2B cells (Fig. 6A). Continuous optical sections showed that the Gene 33 signals were detectable in all sections (Fig. 6B). Taken together, our data demonstrate that Gene 33 is partially localized in the nucleus, the 14-3-3BD regulates the nuclear localization of Gene 33, the EBD regulates the chromatin localization of Gene 33 and c-Abl, and the putative NLS is not important for the nuclear localization of Gene 33.

Gene 33 binds to histone H2AX and promotes interaction between ATM and histone H2AX

To investigate the mechanism underlying the activation of DDR by Gene 33, we surveyed a number of components of DDR and repair machinery using co-IP in BEAS-2B cells. We found that both wild-type Gene 33 and Gene 33YA strongly interact with histone H2AX (Fig. 7A). These data are consistent with the observation that Gene 33 is associated with the chromatin fraction of the cells and implies that Gene 33 may directly interact with the DNA damage and repair apparatus.

Given that Gene 33 promotes DDR in an ATM-dependent fashion, that Gene 33 appears to elevate the activity of ATM, and that Gene 33 interacts with histone H2AX (Figs. 2 (D–F) and 7A), we asked whether Gene 33 could interact with ATM

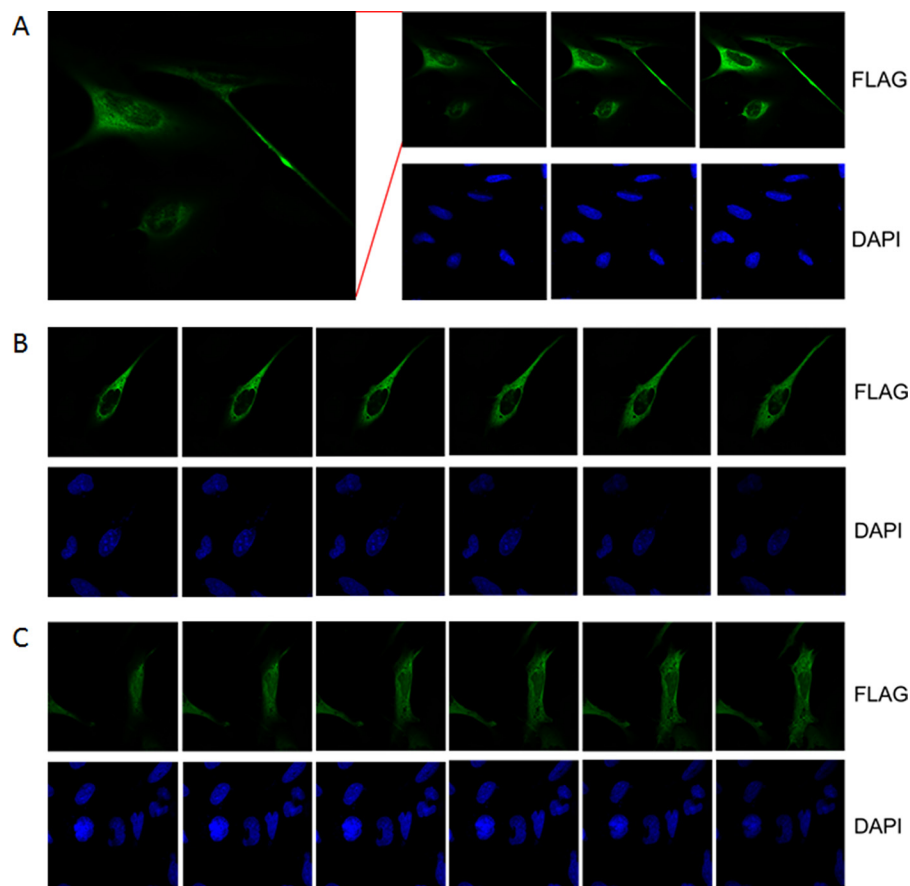


Figure 5. Confocal images showing nuclear localization of ectopically expressed Gene 33. BEAS-2B cells were transfected with the plasmid containing the FLAG-tagged Gene 33 and subjected to confocal imaging as described under “Experimental procedures.” *A*, confocal images showing three continuous optical sections. *B* and *C*, confocal images showing six continuous optical sections of two individual cells.

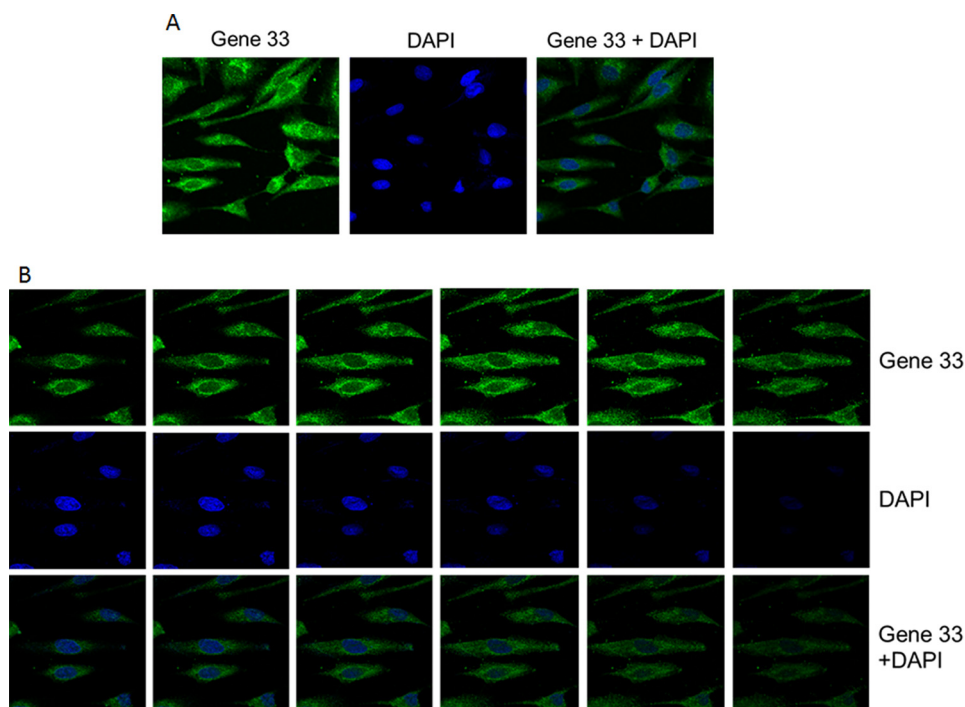


Figure 6. Confocal images showing nuclear localization of endogenous Gene 33. BEAS-2B cells were cultured on 2-well chamber slides and subjected to confocal imaging as described under “Experimental procedures.” *A*, confocal images showing endogenous Gene 33. *B*, confocal images showing six continuous optical sections.

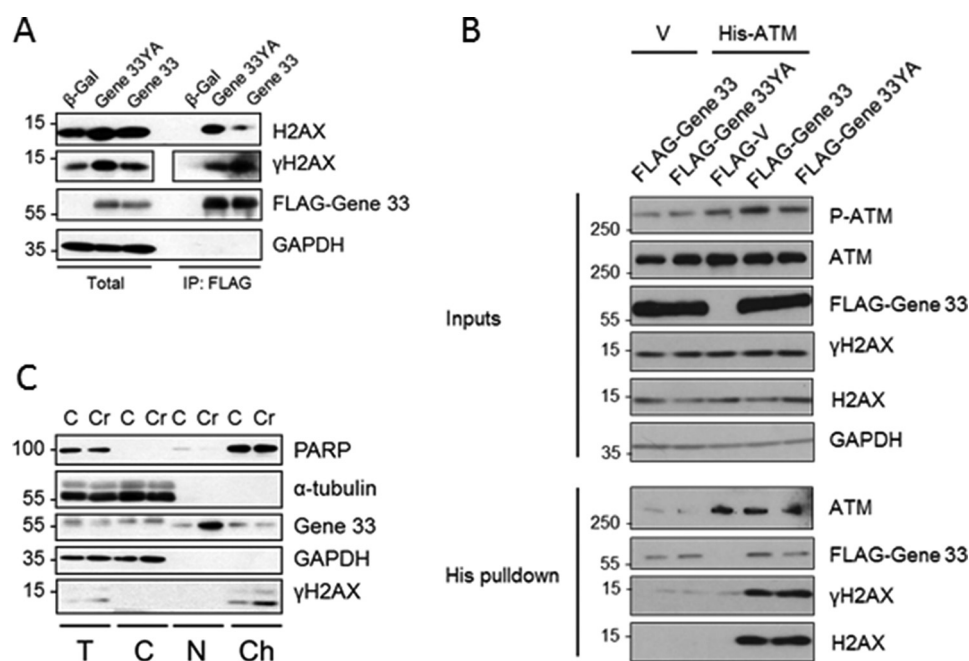


Figure 7. Gene 33 interacts with histone H2AX and promotes interaction between ATM and histone H2AX. A, BEAS-2B cells were infected with adenoviral vectors expressing β -Gal, FLAG-tagged Gene 33YA, or FLAG-tagged Gene 33. Cells were harvested 24 h later for immunoprecipitation with an anti-FLAG antibody. The total lysates and immunoprecipitants were then subjected to Western blotting with the indicated antibodies on the right. B, HEK293T cells were transfected with expression plasmids encoding either FLAG-tagged wild-type Gene 33 or Gene 33YA with or without the presence of His-tagged ATM and harvested 48 h later for the His-pulldown assay with Ni-NTA resins as described under "Experimental procedures." The total lysates and His-pulldown proteins were then subjected to Western blotting to detect the proteins indicated on the right. C, the cell fractionation assay was performed with BEAS-2B cells with or without treatment with 5 μ M sodium chromate (Na_2CrO_4) for 6 h.

and/or affect the interaction between ATM and histone H2AX. We co-transfected His-tagged ATM plasmid along with either the FLAG-tagged wild-type Gene 33 or Gene 33YA plasmids in HEK293T cells, followed by His pulldown with Ni-NTA resins under native conditions. As shown in Fig. 7B, both wild-type Gene 33 and Gene 33YA dramatically enhanced the interaction between His-ATM and histone H2AX. Note that very little histone H2AX was pulled down from cells expressing Gene 33 or Gene 33YA alone or His-ATM alone (Fig. 7B, lanes 1–3 from the left), suggesting that the observed effect of Gene 33 on ATM–histone H2AX interaction was specific. Interestingly, His pulldown did not show proportional increase of Gene 33 with ATM and histone H2AX (Fig. 7B, third panel from the bottom). In addition, we were unable to detect direct interaction between ATM and Gene 33 in IP experiments with endogenous proteins using the anti-Gene 33 antibody or ectopically expressed FLAG-tagged Gene 33 using an anti-FLAG antibody (data not shown). These results suggest that whereas Gene 33 promotes the interaction between ATM and histone H2AX, it may not act by directly bridging the interaction of these two proteins. It is also possible that the interaction between Gene 33 and ATM is transient or too weak to be detected under our experimental conditions.

We next asked whether nuclear localization of Gene 33 is regulated in response to the genotoxic stress. We carried out the cell fractionation assay using BEAS-2B cells with or without treatment with Cr(VI), a known genotoxic agent. As indicated in Fig. 7C, Cr(VI) treatment for 6 h led to a significant increase of Gene 33 in the nuclear fraction. Note that Cr(VI) treatment led to an increased level of γ H2AX. Intriguingly, however, the chromatin fraction of Gene 33 was not elevated by Cr(VI).

Nonetheless, these data indicate that a fraction of Gene 33 is able to translocate to the nucleus upon Cr(VI) treatment.

We noticed that endogenous Gene 33 in the nuclear and chromatin fractions migrated slightly faster than that in the cytoplasmic fraction and the total lysate (Figs. 4H and 7C). A closer examination revealed that faster-migrating Gene 33 also exists in the total lysate but not in the cytoplasmic fraction (Fig. 7C, compare lanes 1 and 2 with lanes 3 and 4). The faster-migrating fraction could be the Gene 33 protein that is not phosphorylated at Ser-250. This would be consistent with the notion that phosphorylation of this residue located in the 14-3-3BD promotes the binding of Gene 33 to 14-3-3, thereby preventing it from entering the nucleus. Alternatively, the short form of Gene 33, which has a deletion of 75 amino acids as a result of alternative splicing (9), may be preferably located in the nucleus. Of note, this deleted portion of Gene 33 does not contain any key domains shown in Fig. 1A (9).

Gene 33 overexpression does not induce DNA damage

The increased interaction between ATM and H2AX as well as the elevated level of DDR after ectopic expression of Gene 33 could be caused by an increase in DNA damage as a result of replicative or genotoxic stresses (43). Alternatively, Gene 33 may elevate DDR without eliciting DNA damage. Previous work showed that ectopic expression of the components of DDR/DNA repair machinery is able to induce DDR in the absence of DNA damage (44). To determine this, we examined whether ectopic expression of Gene 33 could induce actual DNA damage (rather than DDR measured by the level of γ H2AX, a surrogate of DNA damage) in A549 cells using the comet assay. As shown in Fig. 8, neither wild-type Gene 33 nor

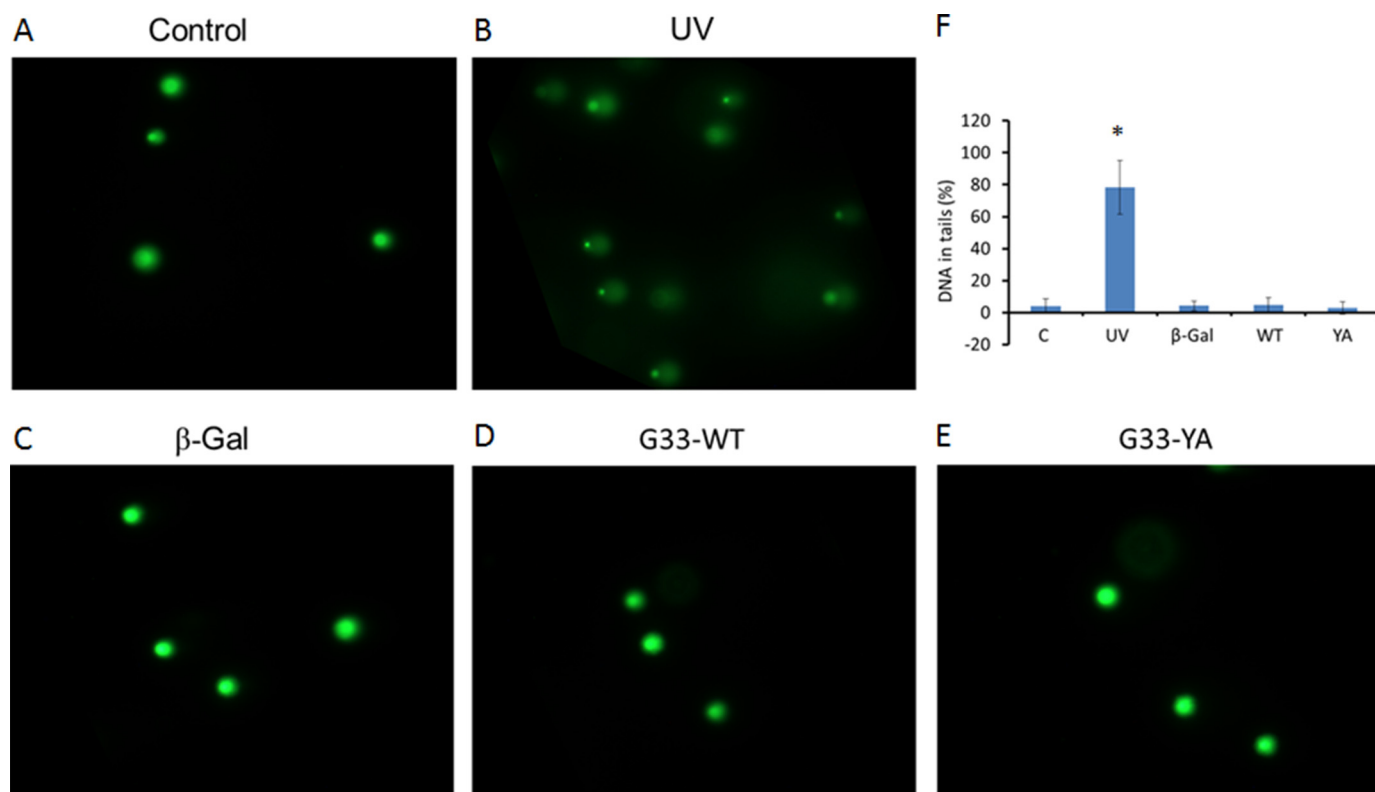


Figure 8. Ectopic Gene 33 expression does not trigger DNA damage. A549 cells were either untreated (*Control*) or treated with UV for 30 s and then recovered for 2 h (*UV*) or infected with the indicated adenoviral vectors at 50 MOI for 24 h. Cells were then used to detect DNA damage by the comet assay as described under "Experimental procedures." A–E, representative images of the comet assay. F, the DNA damage index (percentage of DNA in the tail) was determined using OpenComet comet assay software (mean \pm S.D. (error bars); *, $p < 0.05$).

Gene 33YA induced significant DNA damage 24 h after ectopic expression in A549 cells, manifested as an inability of these proteins to increase comet formation. These data indicate that rather than triggering DDR by inducing DNA damage, Gene 33 promotes DDR without causing DNA damage.

Discussion

The data presented herein support the following conclusions. 1) Ectopic expression of Gene 33 triggers DDR in an ATM-dependent fashion and in both *c-Abl*-dependent and -independent manners in lung epithelial cells and lung cancer cells. 2) Gene 33 promotes apoptosis through both the *c-Abl*/p73 and the EGFR/AKT pathways in lung epithelial cells and lung cancer cells. 3) Gene 33 is partially located in the nucleus and associated with chromatin. 4) An intact EBD motif of Gene 33 is required for Gene 33 to induce apoptosis and is important for Gene 33 to target *c-Abl* to chromatin. 5) Binding to 14-3-3 through its 14-3-3BD prevents Gene 33 from entering the nucleus. 6) Gene 33 interacts with histone H2AX and enhances ATM–histone H2AX interaction, thereby elevating DDR without causing DNA damage. To our knowledge, this is the first report on the nuclear localization and function of Gene 33. These findings are in contrast to the current view that Gene 33 is an exclusively cytoplasmic protein that performs its functions by interacting with other cytoplasmic proteins.

The 14-3-3BD of Gene 33 is clearly important for its nuclear translocation, as a point mutation at the key serine residue (S250A) within the 14-3-3BD dramatically reduced the cyto-

plasmic level and enhanced the nuclear presence of Gene 33 (Fig. 4B). This residue has been shown to be a target of Chk1 (39, 41), a protein kinase involved in cell cycle check point control in response to replicative DNA damage. However, Gene 33 is believed to be phosphorylated by cytoplasmic fraction of Chk1, thereby enhancing its stability by binding to 14-3-3 (39, 41). Our results suggest that binding to 14-3-3 probably prevents Gene 33 from entering the nucleus and show that Chk1 could indeed affect Gene 33 nuclear translocation by phosphorylating its 14-3-3BD (Fig. 4). It is surprising that the putative NLS of Gene 33 appears to be unimportant for its nuclear localization. A similar phenomenon also exists with the tumor suppressor PTEN. Although lacking a classic NLS, its nuclear translocation is mediated by mechanisms such as simple diffusion, active shuttling, and monoubiquitination-dependent nuclear import (45–47). It is conceivable that Gene 33 may enter the nucleus in a similar fashion.

It is interesting that the EBD motif of Gene 33 appears to regulate its chromatin association, as this is also the critical domain for its interaction with EGFR and *c-Abl* (8–10, 14). Our data also show that the EBD helps target *c-Abl* to chromatin, as a higher level of ectopically expressed wild-type Gene 33 in chromatin was accompanied by a significantly elevated level of *c-Abl* at chromatin compared with Gene 33YA (Fig. 4G). This mechanism is probably responsible for the *c-Abl*-dependent activation of DDR and apoptosis by Gene 33 discussed below.

The finding that ectopic Gene 33 expression activates DDR confirms the involvement of Gene 33 in DDR proposed in our recent publication (26), in which we reported that Gene 33 regulates Cr(VI)-induced DNA damage. The nuclear and chromatin localization of Gene 33 described herein provides a spatial explanation for the regulation of DDR by Gene 33, as most of the molecular processes mediating these activities occur in the nucleus. The findings that Gene 33 activates DDR in an ATM-dependent fashion and by promoting interaction between histone H2AX and ATM logically explain why overexpression of Gene 33 triggers DDR. The enhanced interaction between ATM and histone H2AX should promote phosphorylation of histone H2AX by ATM. However, the apparent lack of significant direct interaction between Gene 33 and ATM suggests that Gene 33 may not bridge the interaction between ATM and H2AX or activate ATM directly. It has been reported that initial ATM activation after DNA damage induced by γ -irradiation is a result of ATM autophosphorylation induced by perturbation of the chromatin structure (48). Given our findings that Gene 33 is localized at chromatin and induces DDR without causing DNA damage, it is conceivable that Gene 33 may activate DDR by altering the chromatin structure.

Previous studies have showed that forced targeting of individual components of DNA repair machinery (such as NBS1, MRE11, MDC1, and ATM) is sufficient to activate DDR without DNA damage (44). These findings support an intriguing possibility that Gene 33 may participate directly in DNA repair. This model would explain our previous observation that depletion of the endogenous Gene 33 also increases DDR and elevate DNA damage induced by genotoxic agents (26). An attractive model would be that Gene 33 overexpression induces DDR (without causing DNA damage), probably by perturbing the chromatin structure, whereas Gene 33 depletion may exacerbate DNA damage, induced by genotoxic agents in particular, by weakening DDR, thereby compromising DNA repair. Biologically, reduced Gene 33 expression as a result of mutation, deletion, or expression suppression may lead to increased DNA damage, genomic instability, and tumorigenesis. This model would be consistent with the tumor-suppressing function of Gene 33. We have shown that Cr(VI) suppresses the expression level of Gene 33 in lung epithelial and lung cancer cells (26). It is conceivable that lowered Gene 33 expression as a result of exposure to Cr(VI) (or other carcinogens) may contribute to tumorigenesis via these mechanisms.

We observed that ectopically expressed Gene 33 induces DDR through both c-Abl-dependent and -independent pathways (Fig. 3A). The dependence on c-Abl apparently requires EBD of Gene 33. This result is logical, as c-Abl can interact with and be activated by Gene 33 via the EBD (14). Our finding that both wild-type Gene 33 and Gene 33YA enhances ATM-histone H2AX interaction suggests that this is probably the mechanism underlying the c-Abl-independent activation of DDR. The c-Abl (or EBD)-dependent activation of DDR was unlikely to be a result of inhibition of the canonical EGFR signaling pathway by the intact EBD, as inhibition of EGFR did not affect DDR in these cells (Fig. 3C). Given that c-Abl is an active participant of DDR and DNA repair (37, 49–52), a more logical explanation would be that EBD of Gene 33 facilitates chromatin

localization of c-Abl, thereby promoting DDR. Of note, c-Abl has been shown to be required for proper activation of ATM and for the early stage of DNA double strand break repair (37, 49). However, given that Gene 33YA often induces γ H2AX at an equivalent magnitude as does wild-type Gene 33 (Fig. 2), the c-Abl-independent pathway is probably the dominant mechanism for induction of DDR by Gene 33. Given the complexity of the DDR and the multifunctional nature of Gene 33, the combined signaling outcome of these two pathways is likely very complex.

Previous studies in different cell types have reported both pro-apoptotic and anti-apoptotic functions of Gene 33 (14, 17, 21–23, 39). Gene 33 has also been shown to have no effect on apoptosis in other studies (24). These results indicate that Gene 33 affects apoptosis in a cell type-dependent manner. We have also observed that whether Gene 33 can induce apoptosis upon ectopic expression is cell type-dependent.⁴ This cell type-dependent role of Gene 33 in apoptosis is probably due to the fact that Gene 33 is an adaptor protein with multiple binding partners, including EGFR, CDC42, Grb2, c-Abl, 14-3-3, and NF- κ B (8, 9, 14, 16, 39, 41, 53). The interaction of Gene 33 with these proteins can generate either pro- or anti-apoptotic signals. For example, Gene 33 has been shown to activate NF- κ B (53), a known anti-apoptotic transcription factor (54), whereas inhibition of EGFR or activation of c-Abl by Gene 33 is pro-apoptotic (14, 17). Because of the potentially complex interactions with multiple cellular processes, the eventual biological outcome of Gene 33 expression may depend on the level of Gene 33 expression, the molecular environment of a particular cell type, and/or the physiological states of the cell. It is also important to stress that Gene 33 typically induces significant apoptosis when overexpressed. There is no evidence so far that Gene 33 is a component of the apoptotic machinery *per se*. Thus, the effect of Gene 33 on apoptosis is probably indirect, by altering the balance between survival and death signals in the cell.

At the molecular level, our data in BEAS-2B and A549 cells support the previous findings in mammary epithelial cells that Gene 33 facilitates apoptosis by binding to and activating c-Abl, thereby elevating apoptosis through the c-Abl/p73 pathway (14). Our finding that pharmacological inhibition of EGFR was sufficient to suppress AKT activity supports the notion that inhibition of the AKT-mediated survival pathway also contributes to Gene 33-induced apoptosis in these cells, as Gene 33 is a well-established inhibitor of EGFR (10). This result is consistent with our previous report that Gene 33 promotes apoptosis by inhibiting EGF-induced activation of AKT (17). Thus, the AKT survival pathway, in concert with the c-Abl/p73 pathway, mediates apoptosis induced by Gene 33 overexpression in lung epithelial and lung cancer cells.

Gene 33 is regarded as a tumor suppressor protein in the lung and has been implicated in lung epithelial cell transformation induced by Cr(VI) (26, 55). The obvious connection of Gene 33 with EGFR signaling has led to much research effort devoted to determining how Gene 33 regulates EGFR-mediated cell transformation, tumor progression, and drug resistance. Research

⁴ C. Li, S. Park, X. Zhang, and D. Xu, unpublished observations.

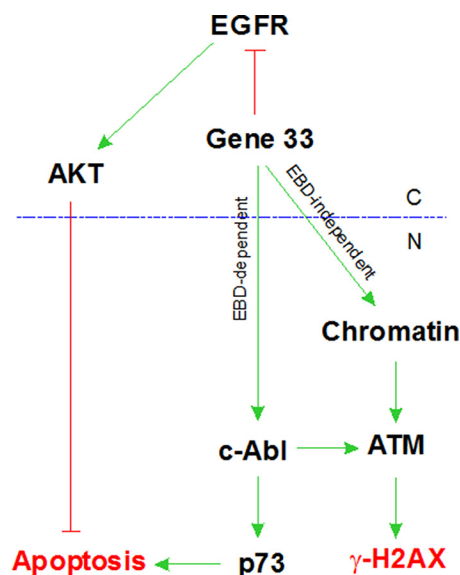


Figure 9. Proposed functions of Gene 33 in DDR and apoptosis. Nuclear Gene 33 activates DDR via both EBD-dependent and EBD-independent pathways by directly activating c-Abl and promoting the interaction between ATM and histone H2AX, respectively. Both pathways are dependent on ATM. Activation of c-Abl by Gene 33 also induces apoptosis in a p73-dependent pathway. Gene 33 promotes apoptosis also by inhibiting EGFR and AKT-mediated pro-survival pathway. Both of these pathways are dependent the EBD motif of Gene 33. C, cytoplasm; N, nucleus.

on how Gene 33 may mediate early cellular events that lead to cell transformation, especially induced by genotoxic environmental carcinogens such as Cr(VI), is lacking. The involvement of Gene 33 in DDR, revealed in this and our previous study (26), provides novel insights into how this protein may modulate carcinogenesis. Future investigation in this direction is clearly warranted. Fig. 9 outlines the proposed signaling model based on the current and previous studies.

Experimental procedures

Cell lines and cell culture

BEAS-2B human bronchial epithelial cells, A549 human lung adenocarcinoma cells, and HEK293T cells were obtained from American Type Culture Collection. Cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 units/ml of penicillin and 100 µg/ml of streptomycin sulfate) under 5% CO₂ conditions. Cells were maintained by passing before they reached confluence (every 2–4 days) unless cell treatments required longer culture time before harvesting for analyses, as indicated in the figures.

Antibodies, plasmids, adenoviral vectors, and chemicals

The antibody to Gene 33 has been described previously (9, 17). The antibody to γH2AX was purchased from Biolegend. Antibodies to GAPDH, EGFR, Mig6, and β-actin were obtained from Santa Cruz Biotechnology, Inc. The antibody to M2-FLAG was purchased from Sigma-Aldrich. The antibody to P-p73 (Tyr-99) was purchased from Abcam. Antibodies to P-p53 (Ser-15), p53, P-ATM (Ser-1981), ATM, H2AX, cleaved caspase-3, PARP1, P-ERK, ERK2, P-AKT, AKT, p73, PY-100, pan-14-3-3, α-tubulin, and c-Abl were purchased from Cell Signaling Technology.

The mammalian expression plasmids encoding FLAG-tagged rat Gene 33 and its deletion mutants have been described previously (9). A tyrosine to alanine point mutation was made at residue 356 of rat Gene 33 to create the FLAG-tagged rat Gene 33Y356A (Gene 33YA) mutant. A serine to alanine point mutation was made at residue 250 of rat Gene 33 to create the FLAG-tagged rat Gene 33S250A (Gene 33S250A) mutant. Point mutations were constructed using a PCR-based approach and confirmed by Sanger sequencing. The mammalian expression vector for EGFR has been described previously (9). pcDNA3-Abl-His₆-FLAG was a gift from Benjamin Turk (Addgene plasmid 52684). pcDNA3.1(+) FLAG-His-ATM WT was a gift from Michael Kastan (Addgene plasmid 31985).

Adenoviral vectors encoding β-Gal and Gene 33 have been described previously (9). Adenoviral vector encoding Gene 33YA was constructed using Gene 33Y356A mutant cDNA and the same method for the Gene 33 adenoviral vector (9).

The EGFR inhibitor AG1478 was purchased from Cayman Chemical. The c-Abl inhibitor nilotinib was purchased from AdipoGen. Caffeine was purchased from Sigma-Aldrich. The ATM kinase inhibitor KU-55933 was purchased from Millipore. The Chk1 inhibitor SB218078 was purchased from Tocris Bioscience.

CRISPR/Cas9-mediated Gene 33 depletion

A double-stranded single-guide RNA oligonucleotide (5'-CACCGCAGTCTGAAGTCTTCTGCTC-3'/5'-AAACGAGC-AGAAGAGTTCAGACTGC-3') was cloned into pX330 vector (Addgene) and transfected to BEAS-2B cells along with a vector expressing GFP (pcDNA3-GFP, at one-tenth of the amount of pX330). Transfected cells expressing high levels of GFP (top 3%) were isolated and seeded individually to 96-well plates 24 h post-transfection using FACS. After clonal expansion, the expression levels of Gene 33 in cell clones were determined using Western blotting. Clones with a dramatically reduced level of Gene 33 were selected and tested for indel formation using the T7E1 assay according to the standard procedure and the primer pair 5'-GAGCCATGGGGAATATGAGG-3'/5'-GAAGATCCCCACGTCCATGAA-3'. The same procedure was carried out using the empty pX330 vector for the WT clones.

Western blotting

A standard Western blotting procedure was using throughout the study. SDS-PAGE was carried out using the minigel system from Bio-Rad after harvesting cells with 1× sample buffer. Proteins in gels were then blotted to PVDF membrane. After blocking with TBST containing 5% nonfat dry milk, the membrane was incubated overnight with primary antibodies diluted with TBST containing 5% BSA using dilutions suggested by the manufacturers. After a thorough wash with TBST, the membrane was further incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature followed by thorough washing with TBST buffer. The signals were developed with an ECL system (Pierce) and detected with X-ray film.

Immunoprecipitation

Cells were lysed in the radioimmune precipitation assay buffer supplemented with a mixture of proteases and phosphatase inhibitors. The lysates were then centrifuged for 30 min at $10,000 \times g$. The supernatants were kept as total cell lysates. One μg of the antibody and 40 μl of protein G-agarose resin (Upstate Biotechnology, Inc.) were then added to 1 mg of each cell lysate and incubated at 4 °C overnight, followed by extensive washing with the lysis buffer. Proteins bound to the resin were then extracted with 5 \times SDS sample buffer and subjected to SDS-PAGE followed by Western blotting with appropriate antibodies.

His-pulldown assay

Cells were lysed in the radioimmune precipitation assay buffer supplemented with proteases and phosphatase inhibitors. The lysates were then centrifuged for 30 min at $10,000 \times g$. The supernatants were kept as total cell lysates. 40 μl of Ni-NTA resins (Thermo Fisher Scientific) were then added to 1 mg of each cell lysate and incubated at 4 °C for 4 h, followed by extensive washing with the lysis buffer. Proteins bound to the resin were then extracted with 5 \times SDS sample buffer and subjected to SDS-PAGE followed by Western blotting with appropriate antibodies.

Cell fractionation assay

Cell fractionation experiments were carried out on ice or at 4 °C throughout. Cells were harvested by trypsinization after they were washed once with ice-cold PBS followed by centrifugation at $100 \times g$ to pellet the cells. Cell pellets were washed once with ice-cold PBS and resuspended in Buffer H (10 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 100 mM NaF, 1 mM sodium orthovanadate, and protease inhibitors). After centrifugation for 8 min at $1500 \times g$, supernatants were transferred to fresh tubes and centrifuged again for 15 min at $14,000 \times g$. The supernatants after the second centrifugation were kept as cytoplasmic fractions. The pellets after the first centrifugation were washed twice with Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitors) followed by centrifugation for 10 min at $1500 \times g$. After discarding the supernatants, the pellets were resuspended in Buffer C (10 mM HEPES, pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% Nonidet P-40, 1 mM DTT, and protease inhibitors) followed by extensive vortexing and centrifugation 15 min at $14,000 \times g$. The supernatants were transferred to fresh tubes as nuclear extracts. The pellets were washed with Buffer C, followed by centrifugation for 10 min at $14,000 \times g$. After discarding the supernatant, the pellets were kept as the chromatin fractions. Forty μg of proteins from each fraction were subjected to SDS-PAGE and Western blotting to detect the indicated proteins.

Confocal microscopy

Cells were cultured on 2-well chamber slides. Twenty-four h later, cells were fixed with 1% formaldehyde followed by 70% ethanol, permeabilized with 0.1% Triton X-100, blocked with

1% BSA, and incubated with either an anti-Gene 33 antibody or an anti-FLAG antibody followed by FITC-conjugated secondary antibody. The slides were embedded in Antifade containing DAPI. The fluorescent images were captured using a confocal microscope.

Comet assay

The alkaline version of the comet assay was performed essentially as described previously (56). Briefly, after treatments, A549 cells were trypsinized and washed with PBS. Then 4000 cells in 40 μl of PBS were mixed with 120 μl of low-melting point agarose in PBS at 37 °C. One hundred ten μl of the mixture was then pipetted onto each well of the Trevigen COMET-SLIDE (Trevigen Inc., Helgerman, CT). After 15 min at 4 °C for solidification, the slides were immersed in prechilled lysis solution (1.2 M NaCl, 100 mM Na₂EDTA, 0.1% sodium lauryl sarcosinate, 0.26 M NaOH) at 4 °C overnight. The slides were then rinsed in the electrophoretic buffer (30 mM NaOH, 2 mM Na₂EDTA, pH \sim 12.3) followed by electrophoresis at 10 V for 25 min in electrophoretic buffer at room temperature. After electrophoresis, the slides were rinsed with distilled water, fixed with 70% ethanol, and air dried. The slides were then stained with Vista Green DNA Dye (Cell Biolabs, San Diego, CA). Images were then acquired using a fluorescence microscope and analyzed with the OpenComet comet assay software to calculate the percentage of tail DNA.

Author contributions—C. L. and S. P. conducted the majority of the experiments and participated in the data analysis; X. Z. and H. Z. conducted some of the experiments; L. M. E. helped with confocal imaging and critically read the manuscript. Z. D. critically read the manuscript. D. X. designed the study, conducted some of the experiments, and wrote the manuscript.

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